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**Protecting Bactofencin A to enable its Antimicrobial Activity using Mesoporous Matrices**

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**Abstract**

There is huge global concern surrounding the emergence of antimicrobial resistant bacteria and this is resulting in an inability to treat infectious diseases. This is due to a lack of new antimicrobials coming to the market and irresponsible use of traditional antibiotics. Bactofencin A, a novel antimicrobial peptide which shows potential as an antibiotic, is susceptible to enzyme degradation. To improve its solution stability and inherent activity, bactofencin A was loaded onto a traditional silica mesoporous matrix, SBA-15, and a periodic mesoporous organosilane, MSE. The loading of bactofencin A was considerably higher onto SBA-15 than MSE due to the hydrophilic nature of SBA-15. While there was no detectable peptide released from SBA-15 into phosphate buffered saline and only 20 % of the peptide loaded onto MSE was released, the loaded matrices showed enhanced activity compared to the free peptide during *in vitro* antimicrobial assays. In addition, the mesoporous matrices were found to protect bactofencin A against enzymatic degradation where results showed that the SBA-15 and MSE with loaded bactofencin A exposed to trypsin inhibited the growth of *S. aureus* while a large decrease in activity was observed for free bactofencin upon exposure to trypsin. Thus, the activity and stability of bactofencin A can be enhanced using mesoporous matrices and these matrices may enable its potential development as a novel antibiotic. This work also shows that *in silico* studies looking at surface functional group and size complementarity between the peptide and the protective matrix could enable the systemic selection of a mesoporous matrix for individual bacteriocins with potential antimicrobial therapeutic properties.

## Introduction

The number of new antimicrobial drugs being brought to the market has declined rapidly over the past decade and this coupled with the non-prudent use of existing antibiotics has led to the emergence of antibiotic resistant bacteria (Cansizoglu and Toprak 2017). There is huge global concern surrounding the emergence of these bacteria as they are resulting in an inability to treat infectious diseases. This will have huge impact on our health services both now and into the future if this problem is not addressed immediately (Cavera *et al.* 2015). It is estimated that antimicrobial resistance (AMR) could cause 10 million deaths a year by 2050 (O'Neill 2014). Many of the old reliable antibiotics are now becoming ineffective and it is thought that there are only two solutions to this problem; repurposing existing therapeutic drugs or the discovery of new antimicrobial molecules. Unfortunately, pharmaceutical companies appear to be more focussed on treatments for metabolic and chronic disease drugs due to the more profitable nature of these products than in developing new antibiotics (Fernandes and Martens 2017).

Antimicrobial peptides are one such biological product that could be a means to tackle this overwhelming issue. There have been a number of antimicrobial peptides identified for their activity against both Gram-positive and Gram-negative bacteria (Hancock and Sahl 2006, O'Shea *et al.* 2013) including *Streptococcus pneumoniae*, staphylococci (including methicillin-resistant *Staphylococcus aureus* (MRSA)), vancomycin-resistant enterococci (VRE), various mycobacteria, and *Clostridium difficile*. These peptides generally have a high potency and low toxicity and have the advantage of being produced by microorganisms or bio-engineered (Cotter *et al.* 2013). They also exhibit anti-viral, anti-fungal and anti-cancer activity but this study will focus on their role as potential antibiotics (Li *et al.* 2012). To-date there is only one antimicrobial peptide mimic in clinical trials called brilacidin. Brilacidin (Medicine 2018), with a molecular weight of less than 1 kDa is currently in phase 2 of clinical trials in the US and has shown activity towards a range of Gram-positive and Gram-negative pathogens including some drug and multidrug resistant strains (Mensa *et al.* 2014). The use of brilacidin to treat acute *S. aureus* skin infections is being reviewed.

A key emerging group of antimicrobial peptides include the bacteriocins which are produced by Lactic Acid Bacteria (LAB). Bacteriocins are ribosomally synthesized hydrophobic peptides which usually display activity towards bacteria closely related to the producer (Lozo *et al.* 2017) and may be subdivided based on whether or not they have undergone post translational modifications (Class I) or

not (Class II). This group of antimicrobial peptides exhibit enhanced potency, are biocompatible and stable and can have a narrow or broad range spectrum of activity (Cotter *et al.* 2013). Bacteriocins are known to possess antimicrobial activity towards pathogens present in the GI tract so could therefore provide an alternative to antibiotics currently used to treat infections in this area (O'Shea *et al.* 2009). In addition, all LAB and their products are generally regarded as safe (GRAS) by the US Food and Drug Administration (FDA) (Acedo *et al.* 2017).

These peptides have a different mode of action when compared with traditional antibiotics therefore minimising the potential for the emergence of antibiotic resistance. There are a number of studies carried out on the antibacterial properties of a range of various bacteriocins including nisin, thuricin CD & lacticin 3147. Nisin and lacticin 3147 exhibit activity against a range of bacterial strains including *S. pneumonia*, MRSA, VRE, various mycobacteria and *C. difficile* and thus are potential therapeutics for hospital acquired infections (Cotter *et al.* 2013, Flynn *et al.* 2018). Nisin, a lantibiotic produced by *Lactococcus lactis* (Okuda *et al.* 2013), is FDA-approved as a food additive (E234) and is certified as a GRAS (Generally Regarded as Safe) excipient. Nisin has been shown to be more effective as a treatment for *S. pneumonia* in mice than vancomycin (Field *et al.* 2010). Thuricin CD is an example of a narrow-spectrum sacitibiotic bacteriocin which targets spore forming Gram-positive bacteria including *C. difficile* (Mathur *et al.* 2017). This bacteriocin has a very narrow spectrum of activity and could be used specifically to treat *C. difficile* infections of the gut. (Acedo *et al.* 2017, Ahn *et al.* 2017, Lozo *et al.* 2017). Enterocin NKR-5-3, a class IIa bacteriocin, shows potent activity against *Listeria* spp. (O'Connor *et al.* 2015) and various bacteriocins have exhibited antifungal properties and can kill spores associated with sporeforming bacteria (Cavera *et al.* 2015). All this highlights the relevance of bacteriocins as clinical therapeutics and as an alternative to traditional antibiotic use. Antimicrobial peptides which display a broad spectrum of activity can cause irregularities to the commensal microbiota of the gut. Therefore bacteriocin therapeutics with narrow spectrum activity could prove more useful. There is evidence that *C. difficile* infections can lead to gut irregularities and the resulting symptoms may need further treatment by antibiotics. In this case a narrow spectrum therapeutic i.e. thuricin CD can target the bacteria without disrupting the resident microbiota (Cotter *et al.* 2013).

Bactofencin A belongs to the Class II lactic acid bacteria group and is a cationic bacteriocin produced by the porcine intestinal isolate *Lactobacillus salivarius* DPC6502. It is a linear peptide of 22 amino acids long that bears a close resemblance to other eukaryotic cationic antimicrobial peptides (O'Shea *et al.* 2013). It is known to exhibit both a narrow spectrum and a broad spectrum of inhibition against a variety of Gram-positive bacteria including *Listeria* spp., *Lactobacillus* spp. and *S. aureus*. One of the advantages to the use of bactofencin A is that it can be produced synthetically (O'Connor *et al.* 2018).

and due to its unmodified nature, controlled large quantities can be generated. It has also been reported by Guinane *et al.*, that the use of a bacteriocin like Bactofencin A can enhance the overall health of the gut microbiota (Guinane *et al.* 2016), a common issue reported after treatment with traditional antibiotics.

However, the use of bacteriocins as antibiotics can be hampered by low solubility, slow dissolution rates, susceptibility to proteolytic enzymes and/or their propensity to aggregate *in vivo* or during formulation and storage (Aguirre *et al.* 2016). Manipulation of these factors at the pre-formulation step or by using delivery systems can help to overcome these issues. There are various delivery systems for large biomolecules, for example the use of oily suspensions, enteric coated tablets, hydrogels, biodegradable nanoparticles and liposomes for oral delivery of peptides and proteins (Jung *et al.* 2000, Watkins and Chen 2015, Moroz *et al.* 2016, Buwalda *et al.* 2017, Meikle *et al.* 2017). The delivery of Octreotide, a synthetic hormone used to regulate the neuroendocrine system using a novel oily suspension with sodium caprylate was investigated and results showed an increase in paracellular permeability in monkeys with this suspension (Tuvia *et al.* 2014). Another peptide, Calcitonin which is frequently administered to regulate calcium homeostasis is generally administered via the parenteral or nasal routes (Moroz *et al.* 2016). Its administration via the oral route has also been investigated using a formulation of enteric coatings containing citric acid. The presence of the acid caused a decrease in the pH of the intestine therefore rendering the resident intestinal and pancreatic enzymes redundant and subsequently permeability was enhanced (Binkley *et al.* 2012). The use of hydrogels as drug delivery systems can protect against the harsh environment (enzymes and low pH) of the GI tract (Qiu and Park 2001). Another gel delivery system, a sodium dodecyl sulfate (SDS)/ $\beta$ -cyclodextrin ( $\beta$ -CD)/chitosan gel has been shown to offer protection to insulin against pepsin *in vitro* (Li *et al.* 2016). Other relevant delivery matrices for encapsulation of peptides include the use of mesoporous matrices (Braun *et al.* 2016, Braun *et al.* 2017, Xie *et al.* 2017, de la Torre *et al.* 2018). Mesoporous matrices have large surface areas and well defined pore structures (Chaudhary and Sharma 2017). They can be designed to have straight narrow channels with high surface areas which can allow for the adsorption of large amounts of various drugs, including proteins and peptides (Hudson *et al.* 2008). These silica matrices contain a large number of hydroxyl groups which may be readily functionalised post synthesis or the matrix can be functionalised using organosilanes, either as bridging or non-bridging precursors, during synthesis. This functionalisation can control the rate of adsorption and release of drugs. The matrices are often structurally stable upon storage and are resistant to heat, changes in pH, mechanical stress and degradation (McCarthy *et al.* 2016). However, the mechanisms of drug release and adsorption depend on the size and chemistry of the adsorbed molecule and the

chemical composition, the pore structure and the pore size of the mesoporous matrix (McCarthy *et al.* 2017). Two mesoporous matrices were investigated during this study, SBA-15 and MSE, as potential carriers for bactofencin A. SBA-15 is a highly ordered hexagonal pure mesoporous silica (Zhao *et al.* 1998), whereas MSE involves using an ethylene-bridged silica precursor to form a mesoporous matrix with ethane groups periodically and homogeneously distributed throughout the pores in an ordered manner (Bao *et al.* 2004). It has been reported that pure silica materials (i.e. SBA-15) are biocompatible, although there may be some issues if they reach the blood stream (Lu *et al.* 2007, Hudson *et al.* 2008). Less is known about MSE because of the lack of *in vitro* and *in vivo* research on this material (Du *et al.* 2016). There have been numerous reports on the loading of mesoporous silicates with various anti-cancer drugs i.e. doxorubicin, topotecan & camptothecin (Luo *et al.* 2014), anti-inflammatory drugs i.e. ibuprofen (Charnay *et al.* 2004) and poorly water soluble drugs i.e. telmisartan (Zhang *et al.* 2010). However fewer studies have been carried out on peptide loading onto mesoporous matrices. A previous study from our group showed that pore size and chemical functionality had an effect on loading and release of nisin A from mesoporous matrices. This study also highlighted the protective nature of a mesoporous matrix against enzymatic degradation of nisin while retaining activity (Flynn *et al.* 2018).

In this present study we investigate the loading of the novel antimicrobial peptide, bactofencin A onto SBA-15 and MSE. In addition the activity of the loaded matrices against *S. aureus*, the biocompatibility of both matrices against Human Embryonic Kidney (HEK293) cells and the role of the matrices in protecting the bacteriocin against proteolytic enzymes found *in vivo* were examined.

## **Materials & Methods**

### **Materials**

*Staphylococcus aureus* culture (DSMZ 20231) was purchased from DSMZ. Human Embryonic Kidneys (HEK293) cells were gifted to our research group by Dr. Pat Kiely's Research Group, Graduate Entry Medical School, University of Limerick. Brain Heart Infusion (BHI) broth and agar, and isopropanol were obtained from Fisher Scientific. Acetonitrile (ACN), trifluoroacetic acid (TFA), hydrochloric acid (37 %), DMEM high glucose media (D6429), fetal bovine serum (FBS), trypsin/EDTA, penicillin/streptomycin, phosphate buffered saline (PBS), proteinase K and trypsin were all obtained from Sigma. MTT cell assay kits were obtained from Merck Millipore (CT02). Synthetic bactofencin A was provided to us from our collaborators in Teagasc Food Research Centre, Moorepark, Cork, Ireland.

### **Synthesis of Bactofencin A**

Synthetic Bactofencin A was used throughout this study. Peptide was synthesised on an H-Cys (Trt)-HMBP pre-loaded resin using microwave-assisted solid phase peptide synthesis (MW-SPPS) performed on a Liberty Blue microwave peptide synthesizer (CEM Corporation, Mathews, North Carolina, USA). Crude peptide was purified using RP-HPLC on a Semi Preparative Jupiter Proteo (4 $\mu$ , 90Å) column (Phenomenex, Cheshire, UK) running a 15-35% acetonitrile 0.1% TFA gradient over 40 minutes where buffer A is Milli Q water containing 0.1% TFA and buffer B is 90% acetonitrile 0.1% TFA. Fractions containing the desired molecular mass were identified using matrix assisted laser deionisation -time of flight-mass spectrometry (MALDI-TOF-MS) on an Axima TOF<sup>2</sup> MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK) and were pooled and lyophilized on a Genevac HT 4X lyophilizer (Genevac Ltd., Ipswich, UK) (O' Connor *et al.* 2018).

To estimate the dimensions of Bactofencin A, calculations using the amino acid sequence with the disulphide bridge were carried out with Materials Studio version 7.0 from Accelrys Inc., and the COMPASS II force field, applicable for organic molecules, including heterocyclic systems (Sun 1998).

#### **Synthesis of SBA-15 and MSE**

SBA-15 and MSE were synthesised and characterised by solid state nuclear magnetic resonance spectroscopy, scanning and transmission electron microscopy, X-ray diffraction, nitrogen adsorption analysis and attenuated reflectance Fourier transform infra-red spectroscopy according to previously published protocols (Sayari *et al.* 2004, Bao *et al.* 2006, Flynn *et al.* 2018).

#### **Adsorption/Release of Bactofencin A onto two Mesoporous matrices (SBA-15 & MSE).**

Samples were UV sterilised x 30 minutes and 5mg of each MPS was added to 1mL of a sterile 0.5 mg/mL solution of bactofencin A (in 50mM sodium phosphate buffer pH 6.8) and mixed until the sample was homogenous. Sterility was maintained from this point forward for activity studies to follow. These mixtures were incubated on a rocker at 37°C x 2 hours. After this incubation time, samples were centrifuged at 5000rpm x 15 minutes. The supernatant was removed and analysed by HPLC to determine the amount of bactofencin A remaining in the supernatant and subsequently the loading concentration of the bactofencin A onto the mesoporous matrices. A control was set up of 1mL of a 0.5mg/mL solution of bactofencin A and this was also incubated for the same time under the same conditions. After the supernatant was removed, the pellets were allowed to dry in a biological safety cabinet (BSC) to maintain sterility. In order to investigate the release of bactofencin A from the MPS, 1mL of sterile PBS was added to the dried pellets in a 1.5mL tube. Solutions were mixed to ensure the pellets were in suspension with the PBS and these were incubated, rocking at 37°C x 24 hours. At each analysis timepoint (2, 4, 6 & 24 hrs), the tube was centrifuged at 5000rpm x 15 minutes,



supernatant was removed, ensuring the MPS/bactofencin A pellet does not come away with the supernatant. The tube was replenished with 1mL of fresh sterile PBS and vortexed until a homogenous mixture was obtained. The supernatant was kept for analysis by HPLC where the amount of peptide released at each timepoint was determined. Pellets were allowed to dry in a BSC after the release assay. Analysis was carried out in triplicate on two random testing days.

#### **HPLC determination of Bactofencin A**

The HPLC determination of bactofencin A concentration was carried out using an Agilent Infinity 1260 HPLC/MS and a Phenomenex C18 Gemini (5 $\mu$ m, 110Å, 250 x 4.6mm) column. The mobile phase used was A: MilliQ H<sub>2</sub>O with 0.1% TFA and B: 90% ACN with 0.1% TFA. A gradient elution was set up as follows: concentration of B increased from 10 to 30% from 5 to 45 minutes. From 45 to 50 minutes the concentration of B increased to 81% and returned to 10% until 55 minutes. 20 $\mu$ l of sample was injected and the flow rate was 0.8mL/minute. The presence of bactofencin A was detected by absorbance at 214nm. Concentrations of bactofencin A and hence % loading and release was calculated as a % of the peak area (RT:~25 mins) of the control.

#### **MIC<sub>50</sub> of Bactofencin A against *S. aureus***

Bactofencin A was dissolved in 50mM sodium phosphate, pH 6.8 at a concentration of 1mg/mL. A microtitre assay using 96 well plates was used to determine an MIC<sub>50</sub> against *S. aureus* (DSMZ 20231). Overnight culture was grown at 37°C, shaking at 250rpm. Microtitre assays were set up in triplicate in 96 well plates as follows: 200 $\mu$ l of BSA (0.1% in PBS) was added to each well and incubated x 30 minutes at 37°C in order to prevent the peptide adhering to the sides of the well (O'Shea *et al.* 2013). After 30 minutes wells were washed with PBS and allowed to dry. Varying concentrations of peptide (0.5-5 $\mu$ g/mL) were added to the test wells first, followed by 50mM sodium phosphate buffer to make up a volume of 50 $\mu$ l. Overnight culture was diluted to an optical density (OD) of 0.1 using BHI broth and monitored at 590nm. 150 $\mu$ l of the diluted culture was then added to each well. A control was set up with 50 $\mu$ l of buffer and 150 $\mu$ l culture. Blanks (media only) were also set up on the same plate. Each sample, blank and control was set up in triplicate. Plates were incubated in a Biotek ELx808 Ultra microplate reader (Mason Technologies, Dublin, Ireland) and OD<sub>590</sub> was monitored with mild shaking before each reading. Readings were taken every 30 minutes over a 24 hour period. Blank readings were subtracted from readings in the test well. MIC<sub>50</sub> was determined as the concentration of bactofencin A required to kill 50% of the bacterial population after 24 hours. Results include two random tests of triplicates.



**Bioactivity of peptide loaded MPS against *S. aureus***

An overnight culture of *S. aureus* was grown up in BHI broth. This culture was diluted to an OD of 0.1 after ~ 16 hours with BHI broth. Similar to the loading study outlined previously, MPS were loaded with bactofencin (0.5 mg bactofencin per 5 mg MPS) for 2 hours. Following this loading period the suspension was centrifuged at 5000 rpm x 10 mins and the supernatant was removed. Pellets were allowed to dry in a biosafety cabinet for 1-2 hours. The dried mesoporous silicate pellets with adsorbed bactofencin A were transferred to 3mL of the diluted bacterial culture and were incubated for 5 hours at 37°C. Incubation time of 5 hours was chosen as this is the time point where decline in bacterial numbers when incubated with the peptide was evident due to nutrient depletion during MIC<sub>50</sub> tests. Controls were set up with (a) bactofencin A (0.5 mg/ 3 mL diluted cell culture) only and (b) MPS (5mg/3mL diluted cell culture) only. After 5 hours, serial dilutions were made in PBS and plated onto BHI plates. Plates were incubated at 37°C for 24 hours and colonies were counted and expressed as colony forming units (CFU) per mL. Results include two random tests of duplicates.

**Enzyme degradation of Bactofencin**

The susceptibility of bactofencin A to degradation by two enzymes was investigated. A 1mg/mL solution of trypsin or proteinase K and a 1mg/mL solution of bactofencin A (50mM Sodium Phosphate pH 6.8) were mixed in a 5: 1 (Peptide:Enzyme) ratio. This mixture was incubated at 37°C with rocking for 24 hours, acidified to pH 2 using 1M HCl to stop the enzyme activity and analysed using HPLC.

**Protection against enzyme degradation**

Bactofencin A was loaded onto both MSE and SBA-15 as described previously. After removal of the supernatant, the pellets were allowed to dry for 1-2 hours under sterile conditions and 200µl of trypsin (1mg/mL) was added to the pellets and allowed to incubate rocking for ~16 hours at 37°C. A control was set up with bactofencin A (1mg/mL) and trypsin (1mg/mL) in the ratio 5: 1 (Peptide:Enzyme) and also allowed to incubate rocking for ~16 hours at 37°C. Following incubation, samples were centrifuged at 5000rpm x 10mins, washed with DI H<sub>2</sub>O and centrifuged again. Supernatant was removed and the pellet was allowed to incubate with 3 mL of *S. aureus* (OD = 0.1) for 5 hours. A control solution of bactofencin A and trypsin (100ul) was also added to 2.9mL of culture for 5 hours. After this time, serial dilutions of test samples and controls were made in PBS and plated onto BHI plates. These plates were allowed to incubate overnight at 37°C and colonies were counted and expressed as CFU/mL. Analysis was carried out in duplicate on two random testing days.

**Biocompatibility of MPS with HEK293 cells using In Vitro cell assays**

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) assays were carried out on mammalian HEK 293 (Human embryonic kidney) cells to ascertain the compatibility of the matrices with mammalian cells. Cells were maintained in DMEM media with 10% FBS, 1% Penicillin/Streptomycin and 1% L-glutamine. Cells were incubated at 37°C, 5% CO<sub>2</sub> and 95% relative humidity. Passage numbers less than 30 were routinely used for the MTT assays. 10,000 cells per well were seeded in a 96 well plate. Cells were incubated overnight to ensure adherence of the HEK293 cells. The matrices were UV sterilised for 30 minutes. Media was changed in the wells before addition of the matrix. A stock suspension of 50mg/mL of matrix in PBS was prepared and sonicated for 1 hour to achieve a homogenous sample. Stock solutions of samples were added to each well to give a final concentration of 1, 3 & 5 mg/mL and the matrix was incubated with the cells for 2, 6 & 24 hours. Live controls with buffer (50mM sodium phosphate pH 6.8) were set up in addition to dead controls which were treated with 10% Triton-X. Blank media with matrices were also set up to ensure the matrices did not interfere with readings. After the incubation period, MTT reagent was added (10µl) to each well and allow to incubate for a further 4 hours. After 4 hours, 100µl isopropanol with 0.1 M HCl was added to each well according to manufacturer's instructions, plates were centrifuged at 1000rpm x 5mins and the supernatant (100 µl) was transferred to a new 96 well plate. Plates were read at 570 nm and test results were expressed as % cells that were viable after treatment when compared with the live control. Analysis was carried out in triplicate on two random testing days.

**Results and Discussion****Adsorption/Release of Bactofencin A onto Mesoporous silicates (SBA-15 & MSE).**

Bactofencin contains 22 amino acids, has a molecular mass of 2,782 Da and contains two cysteine residues that form an intramolecular disulfide bond. It is a cationic peptide with an isoelectric point of 10.59 (O'Shea *et al.* 2013). This means that it will have an overall positive surface charge in solution at pH values less than this. While the crystal structure of bactofencin is not yet known, using Materials Studio to estimate its size, the largest possible dimensions of bactofencin are 22 Å x 27 Å x 30 Å.

Both mesoporous matrices used in this study (SBA-15 and MSE) were characterised extensively by our group in a recent publication (Flynn *et al.* 2018). Both matrices have ordered hexagonal pore structures with pore sizes of 68 Å for SBA-15 and 60 Å for MSE. Given the predicted size of bactofencin A, it should fit inside the pores of both of these matrices. SBA-15 and MSE have isoelectric points of 3.7 and 4.8 (Flynn *et al.* 2018). Therefore, at pH 6.8 these matrices will be negatively charged. MSE contains additional hydrophobic ethane groups in its framework as well as hydrophilic silanol groups

and thus can enable adsorption through hydrophobic or hydrophilic interactions. SBA-15 however contains predominantly hydrophilic silanol groups on its surface with a small percentage of the slightly more hydrophobic siloxane bridges and thus hydrophilic interactions would be expected to dominate. Thus if electrostatic interactions dominate during adsorption, bactofencin would adsorb onto both SBA-15 and MSE. Bactofencin is composed of 10 hydrophilic amino acids and 5 hydrophobic amino acids. While we cannot estimate which of these amino acids are exposed on the surface of the peptide without its tertiary structure, the higher percentage of hydrophilic groups would suggest that it might bind preferentially to SBA-15 than MSE. In our previous study, nisin, which contains a high proportion of hydrophobic residues, bound preferentially to MSE over SBA-15 (Flynn *et al.* 2018).

From the adsorption studies, bactofencin A loading was indeed found to be considerably higher onto SBA-15 than MSE, giving a loading of 93ug and 34ug of bactofencin respectively per mg of MPS (Figure 1). The lower loading onto MSE may be due to the hydrophobic nature of the MSE material. It has been reported that enhanced loading is possible when the pore size of a mesoporous silicate (MPS) is slightly larger than the protein/peptide and when the charges on the MPS and the peptide are complimentary (Deere *et al.* 2003). Deere *et al.* studied the loading of cytochrome C onto MPS and a pore size slightly larger than the protein and the complimentary surface charges of the protein and the matrix were vital for high loading. Functionalisation of the mesoporous matrix with more hydrophobic groups did not enhance loading of the peptide, indicating that bactofencin preferentially adsorbs to a more hydrophilic surface and hydrogen bonding or electrostatic interactions dominate over hydrophobic interactions. Unfortunately complete adsorption isotherms could not be generated due to a limited supply of bactofencin A.

When the release of bactofencin A from both MPS was monitored into PBS using RP-HPLC over 24 hours, no release was observed from SBA-15. As discussed previously, bactofencin A is a cationic peptide and the presence of the negatively charged –OH groups in the SBA-15 may result in strong electrostatic interactions between peptide and the material preventing leaching of the bactofencin A. There are less OH groups present on the surface of MSE (Flynn *et al.* 2018) so this indicates a lower degree of electrostatic interactions between peptide and material and therefore a reduced loading, a weaker binding and thus an increased rate of release were observed. A burst release of bactofencin A from MSE was observed up to 6 hours and 20 % or ~ 6ug/mL of the adsorbed bactofencin was released in total (Figure 2a & b). The release of the peptide from the matrix is through diffusion through the ordered pores due to the insoluble nature of the matrices at neutral pH (Balas *et al.* 2006). The loading of a number of antibiotics on MPS has been studied in the past and the efficacy of SBA-15 silica-based bioceramics as a drug delivery system with vancomycin, rifampicin and linezolid, either alone or in

combination has been confirmed and controlled antibiotic release was achieved (Molina-Manso *et al.* 2012).

#### ***MIC<sub>50</sub> of Bactofencin A against S. aureus***

During this study the antibacterial effect of bactofencin A against *S. aureus* was examined. The MIC<sub>50</sub> with *S. aureus* was determined to be 5ug/mL (Figure 3). It has been reported in a previous study by O' Shea *et al.* (O'Shea *et al.* 2013) that bactofencin concentrations of 1-5uM were sufficient to inhibit the growth of *S. aureus*. These concentrations equate to the range 14-28ug/mL but in this work, much lower bactofencin A concentrations have similar outcomes. Bactofencin A contains a large percentage of the basic residues arginine and lysine at the N terminal and it is thought that this basic charge on the peptide allows for electrostatic interactions with the negatively charged bacterial cell membrane (O'Shea *et al.* 2013), therefore causing inhibition of bacterial growth. Other bacteriocins, e.g. nisin A and lacticin 3147, have shown similar trends against *S. aureus* and other Gram-positive bacteria

#### ***Bioactivity of peptide loaded MPS against S. aureus***

The level of bacterial growth was greatly inhibited when the bactofencin A loaded SBA-15 & MSE (loaded with 0.47 mg and 0.17 mg of bactofencin respectively) were incubated with a culture of early log phase *S. aureus*, by 87% and 69%, respectively (Figure 4). This was a higher antimicrobial activity than the free bactofencin control which contained 0.5 mg of bactofencin and inhibited the same culture by only 51%. This would suggest that the bactofencin A loaded onto the matrices is more active than free bactofencin A. This enhanced activity could be due to the presence of some of the adsorbed peptide on the outer surface area of the matrices, making more of the peptide available and/or due to increased contact time between bactofencin A and the bacteria. It is also feasible that in the environment of the culture, bactofencin is deactivated to some extent, accounting for the lower activity of the free bactofencin in the control compared to the bacteriocin adsorbed onto the matrices. While a greater inhibition was noted with SBA-15 loaded particles over MSE loaded particles, this may be due to the higher loading of bactofencin in this sample. Taking the loading into account, the greatest antimicrobial activity per mg of bactofencin present was observed for the MSE/bactofencin A sample. MSE or SBA-15 by itself demonstrated no antimicrobial activity against *S. aureus*. Bactofencin A releases into PBS from the MSE matrix but not from SBA-15 but it is feasible that it is released from both matrices in the media used in the activity assay. This may happen at a faster rate from MSE, where the interaction between bactofencin and its surface appears to be weaker and results in a higher specific activity.

### ***Protective nature of MPS against proteolytic degradation of Bactofencin A***

In order to probe if the mesoporous matrix of SBA-15 and MSE does indeed protect bactofencin A from degradation, a proteolytic degradation study was conducted. The proteolytic degradation of proteins and peptides is one of greatest stumbling blocks to their therapeutic use. Degradation by the serine endopeptidases trypsin, chymotrypsin and elastase is a huge issue surrounding the oral delivery of peptides or proteins. Ordered porous silicates used as drug delivery matrices have been shown to protect antimicrobial peptides and enzymes from proteolytic degradation (Kim *et al.* 2007, Braun *et al.* 2016) and therefore maintain their antimicrobial properties. During this study we investigated the role of trypsin on the cleavage of bactofencin A. Trypsin cleaves at the C terminal of arginine and lysine residues and as ~30% of bactofencin A is made up of these basic residues, this peptide is highly susceptible to degradation (Olsen *et al.* 2004). The use of delivery matrices to protect these biomolecules *in vitro* is one method which may be used to overcome this issue. Results in the present study demonstrated that bactofencin A is susceptible to degradation by the luminal proteases trypsin and proteinase K (Figure 5). HPLC analysis shows significant differences in the profile of free bactofencin A and digested bactofencin A. The intact peptide peak was no longer present after digestion with either trypsin or proteinase K, but rather replaced by numerous smaller peaks. When free bactofencin A (not adsorbed to a porous matrix) was exposed to trypsin and digested it only inhibited growth of *S. aureus* by 8% whereas undigested bactofencin A inhibited growth by 51% (Figure 4 & 6). Therefore digestion by trypsin rendered the peptide almost totally inactive. SBA-15 and MSE with loaded bactofencin A exposed to trypsin inhibited the growth of *S. aureus* by 64 and 29% respectively (Figure 6) while the activity of the undigested bactofencin A/SBA-15 and bactofencin A/MSE samples inhibited growth by 87 % and 69 % respectively. SBA-15 appears to offer the highest degree of protection for bactofencin, most likely due to its slow release of the peptide. The bacteriocin may lodge inside the pore structure of the mesoporous matrices which the protease is too large to access. The estimated dimensions of trypsin are 51Å x 41Å x 41Å (Mallen and Hudson 2017) (Guinane *et al.* 2016) which is similar size to the pores of both SBA-15 and MSE, therefore trypsin is most likely to block the pores of the matrix and will be unable to diffuse far down the pores of SBA-15 or MSE to where the bactofencin A is adsorbed. It may also be protected as long as it is absorbed on the surface of the matrix through steric hindrance of the protease to the target basic residues in the bacteriocin. Thus it has been shown how the use of mesoporous silicates (unfunctionalised and organo-bridged) as delivery matrices protects the peptide from proteolysis while preserving and possibly enhancing its antibacterial properties.

### ***Biocompatibility of mesoporous matrices***

The biocompatibility of the matrices was examined using HEK293 cells. Cells were grown in the presence of increasing concentrations of the MPS (1, 3 & 5 mg/mL) for 3 different incubation times (2, 6 & 24 hours). MTT results revealed slight differences in biocompatibility between the two materials. The concentrations of SBA-15 tested showed ~ 10% decrease in cell viability after 2 hours incubation at the higher concentrations tested whereas after 6 hours incubation there was evidence of cell proliferation as cell viability exceeded 100% when compared to the control (Figure 7). Decrease in cell viability after exposure to MSE was slightly higher, where ~ 30-40% decrease in cell viability was evident after 2 hours. However, viability of HEK cells after 6 & 24 hours was >80%. Similar results for the biocompatibility of SBA-15 were seen by Hudson *et al.* where the viability of macrophages exposed to various different unfunctionalised and functionalised MPS decreased by ~20% or less. However % cell viability after exposure to the mesoporous matrices with mesothelial cells was much lower ( $\leq 40\%$ ) at the highest concentration tested (0.5mg/mL) (Hudson *et al.* 2008). Concentrations of SBA-15 tested during that study were 10 fold lower compared to this work. Overall MSE appeared to have a slightly more cytotoxic effect on cell survival than SBA-15. The biocompatibility of these organo-bridged matrices has not been previously reported but the data shown here would suggest that the introduction of bridged organic species has some effect on cell viability. It's widely reported that these mesoporous materials are biocompatible both *in vitro* and *in vivo* and once their function has been exhausted, they can be secreted by the body (Tang *et al.* 2012). Similar results on the biocompatibility of SBA-15 were reported previously where cell (macrophage) survival decreased (down to 80%) at concentrations of 0.3 and 0.5mg/mL after 3 days incubation. However, during this study cell survival fell below this value under the same conditions for mesothelial and C2C12 muscle cells (Hudson *et al.* 2008). Other studies also confirm the biocompatible nature of various MPS matrices but size, surface properties, shape and structure all influence its cytotoxic properties (Tang *et al.* 2012, Esquivel *et al.* 2014, Du *et al.* 2016). After 24 hours for all concentrations tested, viability returned to ~80% and therefore no prolonged damage to cells was noted.

## Conclusion

Bactofencin A could potentially be used a new antibiotic that could be brought to the market. This study demonstrates that by protecting bactofencin A in a mesoporous matrix, its inherent activity against *S. aureus* was improved. In addition, upon exposure to a protease, bactofencin A retained far more activity when in a mesoporous matrix compared to as a free peptide in solution. Bactofencin A was found to preferentially bind to a more hydrophilic surface than a hydrophobic surface but in previous studies with a more hydrophobic bacteriocin, nisin, preferential adsorption was observed to a more hydrophobic surface. This study highlights the possibility of oral delivery of peptides using a

traditional and an organo-bridged mesoporous silicate due to high drug loadings, enhanced antibacterial properties, biocompatibility and enzymatic stability. The anaerobic environment in the gut varies hugely from *in vitro* tests where all assays are performed in aerobic conditions, so it is unclear how the administration of antimicrobial peptides may affect overall microbial gut balance. Therefore *in vivo* testing would be recommended as the next step to this development. While the use of these delivery matrices is promising, *in silico* studies looking at surface functional group and peptide and pore size complementarity between the peptide and the protective matrix would enable a systemic selection of a mesoporous matrix for an individual bacteriocin with potential antimicrobial therapeutic properties.

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### Figure list

**Figure 1** Figure 1. (a) % of bactofencin A loaded onto MSE (grey bar) & SBA-15 (black bar) after 2 hours incubation.

**\*Figure 2.** (a) % and (b) concentration ( $\mu\text{g/ml}$ ) of bactofencin A released from MSE over 24 hours into PBS. Results are average of duplicate on two random testing days with standard deviation. No release of bactofencin A was detected from SBA-15.

**Figure 3.**  $\text{MIC}_{50}$  for bactofencin A against *S. aureus* at concentrations 5, 3, 2, 1 & 0.5  $\mu\text{g/ml}$ , compared to a control (no peptide). All values are means of 6 values  $\pm$  standard deviation. Bactofencin A was incubated with *S. aureus* culture in a 96 well plates for  $37^\circ\text{C} \times 24\text{hours}$ . Results include averages of two random tests of triplicates with standard deviations.

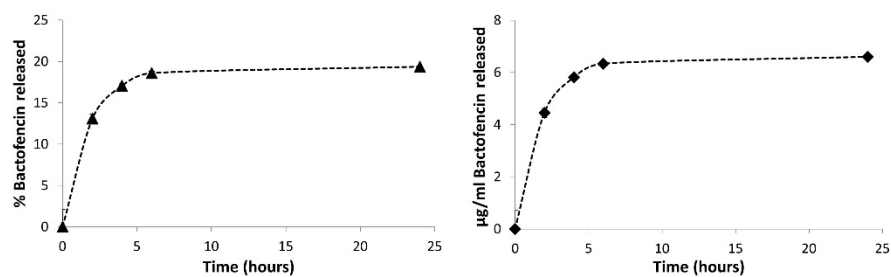
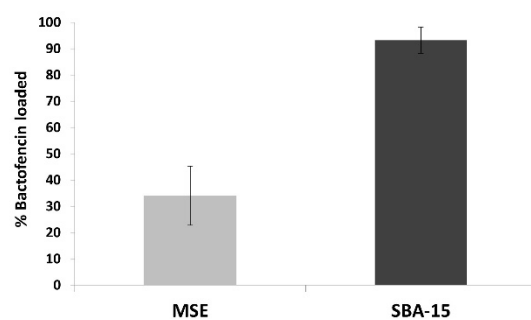
**Figure 4.** Inhibition of *S. aureus* growth by Bactofencin A loaded SBA-15 (5.47 mg added, 0.47 mg bactofencin) and MSE (5.17 mg added, 0.17 mg bactofencin) and free Bactofencin A (0.5 mg) after 5 hours incubation. Results include averages of two random tests of duplicates with standard deviations.

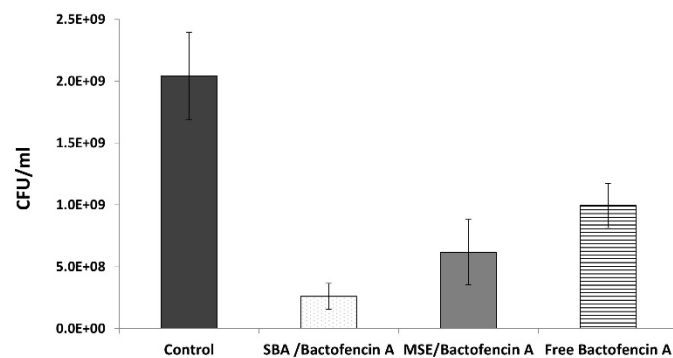
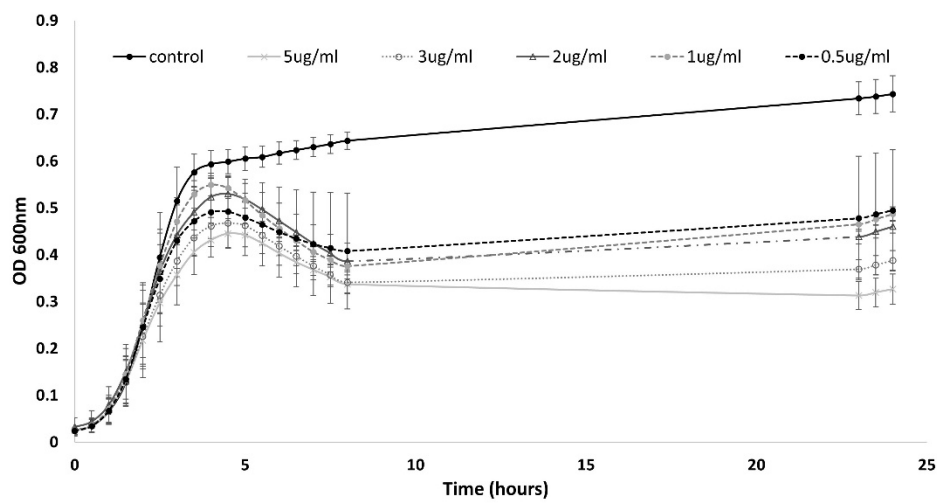
**Figure 5.** HPLC chromatogram of Bactofencin A (0.8mg/ml) and Bactofencin A (0.8mg/ml) incubated with (a) trypsin and (b) proteinase K overnight at  $37^\circ\text{C}$ .

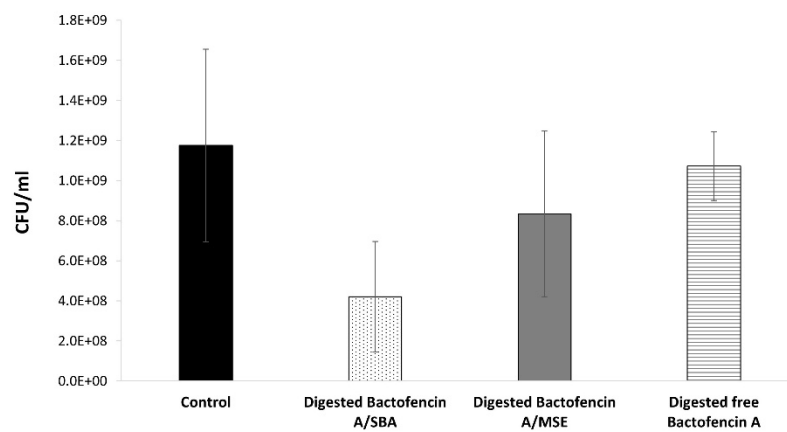
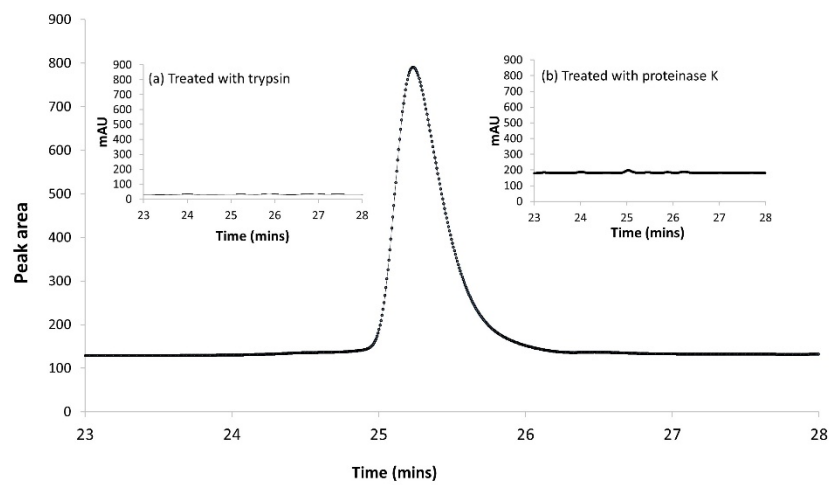
**Figure 6.** Activity of bactofencin A loaded onto SBA-15 (dotted bar) & MSE (grey bar) & free bactofencin A (striped bar) treated overnight with trypsin against *S. aureus*. Results are expressed in CFU/ml. Control was set up with culture only (black bar). Results include averages of two random tests of duplicates with standard deviations.

**Figure 7.** % Viability of Human Embryonic Kidney (HEK293) cells when incubated for up to 24 hours with SBA-15 and MSE. Live controls were set up and results were expressed as 100%, all other results were normalised to the live controls.

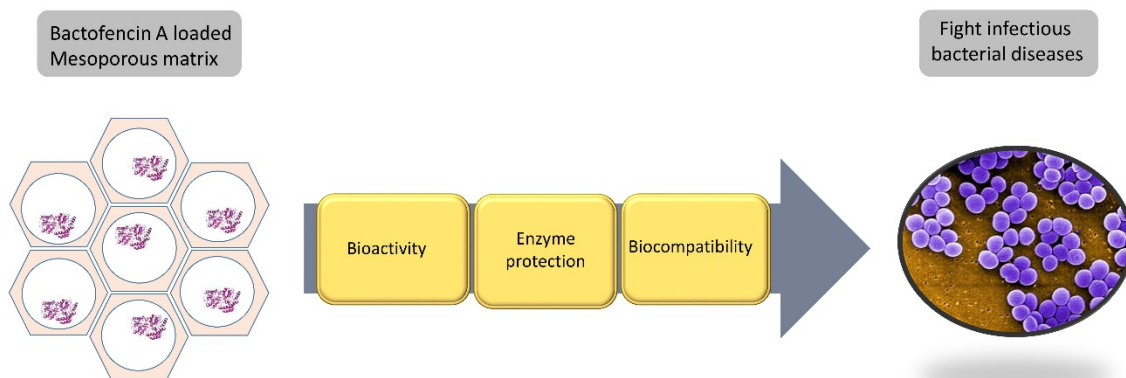
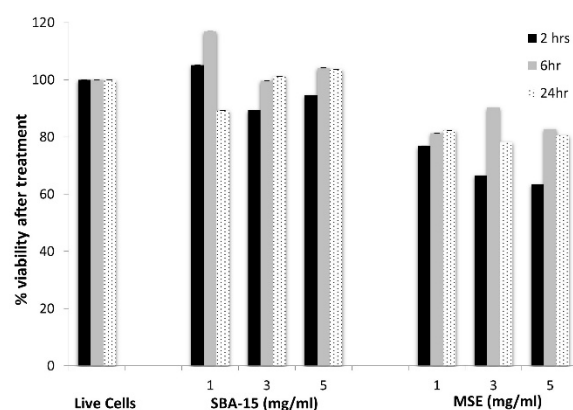
\*All single column fitting except for figure 2 which is 2-column fitting.











## Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

ACCEPTED MANUSCRIPT